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(21) International Application Number: PCT/GB95/00409 (22) International Filing Date: 28 February 1995 (28.02.95) (30) Priority Data: 94301524.8 1 March 1994 (01.03.94) EP (34) Countries for which the regional or international application was filed: GB et al. (71) Applicant (for all designated States except US): AMERSHAM INTERNATIONAL PLC [GB/GB]; Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): STEWART, Gordon, Sydney, Anderson, Birnie [GB/GB]; 14 James Avenue, Loughborough LE11 0QL (GB). DENYER, Stephen, Paul [GB/GB]; 34 Cranedown, Lewes, East Sussex BN7 3NA (GB). JASSIM, Sabah, Abdel, Amir [GB/CA]; University of Guelph, Ontario Agriculture College, Dept. of Food Science, Dairy Microbiology, Guelph, Ontario N1G 2W1 (CA). (74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: SELECTIVE VIRUS CULTURE (57) Abstract A method of obtaining from a virus population comprising a test virus improved virus progeny of the test virus, which method comprises the steps of: (i) providing a target cell sample which comprises target cells of the test virus; (ii) incubating a mixture of a sample of the virus population with the target cell sample so as to effect attachment to and infection of at least one target cell by at least one test virus particle, the conditions being controlled in a manner to ensure that a desired proportion of the test virus does not infect the target bacteria; (iii) incubating the target cells to complete infection by the test virus and to cause virus progeny to be released; and recovering the virus progeny. In a preferred embodiment, an additional step performed between steps (ii) and (iii) comprises neutralising the extracellular virus with an antiviral agent.		

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SELECTIVE VIRUS CULTURE.

This invention relates to infection of host cells by virus particles and selection of virus progeny which have an improved ability to infect host cells compared to the natural virus population.

5 Bacteriophage host interactions are dependent on the binding of tail proteins to specific bacterial surface receptors (Pelczar et al 1993). The absence of such receptors or the infrequent expression of such receptors would render a bacterium resistant
10 (or partially resistant) to bacteriophage infection. Similarly, failure to correctly assemble tail fibres would prevent efficient infection and it may be expected that there would be a Poisson type distribution of tail assembly efficiency giving, in
15 any population of phage, some that attach rapidly and some weakly, if at all. This distribution would not, from current genetic understanding, be expected to reflect differences in the phage genome but simply the phenotypic expression and assembly of protein during
20 phage propagation. Another reason for not suspecting genetic variations as the cause of differences in infectivity is that it may be disadvantageous for viruses to become more efficient at infecting; selection of better attaching virus may result in
25 faster and more widespread infection and killing of host cells, making it harder for virus to survive.

Taking a phage that attaches very efficiently and growing from it a new population of bacteriophage would therefore be expected to give an
30 identical distribution of good and poorly attaching phage superimposable on the original Poisson distribution. In the past it has not been possible to test this hypothesis since it has been impossible to isolate rapidly infecting phage from the rest of the
35 population.

The bacteriophage protection technique,

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described in WO92/02633, allows for the complete destruction of bacteriophage that fail to enter the protective environment of a host bacterial cell within a specified time interval. It is now possible, therefore, to develop bacteriophage replication
5 plaques from only those phage that rapidly attach to and replicate in a host bacterium. The progeny of such phage do not show a distribution of attachment equivalent to the original population but instead the
10 entire population has a greatly enhanced efficiency of host attachment. A repetitive cycle of this technique can lead to a phage population with an entirely altered host affinity. For example, a phage population that might have previously provided only
15 very weak infection of a host can be developed to a population giving a plaque forming unit (pfu) efficiency of 1 and a rapid replication rate. In this way new viral agents, different from those that could be obtained directly from nature, can be constructed.

20 The invention therefore provides a method of obtaining from a virus population comprising a test virus improved virus progeny of the test virus, which method comprises the steps of:

- (i) providing a target cell sample which
25 comprises target cells of the test virus;
- (ii) incubating a mixture of a sample of the virus population with the target cell sample so as to effect attachment to and infection of at least one target cell by at least one test virus particle, the
30 conditions being controlled in a manner to ensure that a desired proportion of the test virus does not infect the target cells;
- (iii) incubating the target cells to complete infection by the test virus and to cause virus progeny
35 to be released; and recovering the virus progeny.

The virus progeny are improved eg. by having

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increased efficiency of infection of the target cell compared with the test virus, which may be due to improved attachment to the target cell, or to any other alteration that results in the virus being better able to attach to and/or infect the cell. The improvement may thus be in the virulence of the virus.

The test virus is preferably a phage and may be a bacteriophage, in which case the target cell is a bacterium. The "target cell" is not necessarily the usual wild type target for the phage; the target cell may be a cell type which is only very rarely infected by the phage in question. For example, the method according to the invention may be performed using phage Felix 01 as the test phage and Salmonella agona, infantis, kubacha, togba and stanley as the target cell. Wild type Felix 01 does not normally efficiently infect these Salmonella species, although it infects other Salmonella species, such as S.typhimurium, but by using the phage breeding method according to the invention, improved Felix 01 can be produced which has the ability to efficiently infect Salmonella agona, infantis, kubacha, togba and stanley producing visible plaques.

In a preferred method according to the invention, an additional step performed between steps ii) and iii) comprises neutralising the extracellular virus with an antiviral agent. Neutralisation of the virus means destroying or killing or any method of inactivating the virus. WO92/02633 describes ways in which this can be achieved, without harming the target cell, for a method involving bacteria/bacteriophage. Neutralisation of extracellular virus may be carried out in other ways known in the art; some methods of neutralisation are demonstrated herein. The particular method used is not material to the invention.

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Although the invention is discussed here primarily in relation to phage and bacterial host cells, the invention also covers breeding of viruses which infect eukaryotic cells, and the resulting
5 progeny virus.

In one embodiment, extracellular phage need not be neutralised chemically. The extracellular phage may simply be separated from the target cells after the desired initial attachment/infection has taken place.

10 Usually, there will be a specified period of time allowed for attachment to and infection of target cells by test phage. The incubation time in step (ii) above may be controlled to ensure partial but incomplete infection of the population. The proportion
15 of test phage present in the mixture which attaches to and infects the target cells is thereby controlled. Physical or chemical conditions may also be altered for this purpose. The conditions and timing required for a particular combination of phage and target cell in the
20 method according to the invention will be readily determined by a person skilled in the art.

The phage breeding method described herein can be performed more than once to produce highly infectious phage. Phage progeny obtained from the
25 first round of breeding are used as test phage in a repeat of the method according to the invention. The second phage progeny will be better able to infect the target cell than the previous phage progeny. Any number of rounds of breeding may be performed. Phage
30 progeny may also be subsequently bred on a different target cell, so as to broaden the range of host affinity.

In a further aspect, the invention provides virus progeny of a test virus, wherein the virus
35 progeny have improved properties compared to the test virus, following one or more rounds of selective

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breeding. The virus progeny may be obtained by the method described therein. Such virus progeny are not found in nature because in nature there is no separation or neutralisation of extracellular virus
5 once the more efficient virus particles have attached to and/or infected the target cell.

Yet another aspect of the invention is a new method of detecting virus infected cells. In the virus breeding procedure, when extracellular virus
10 particles have been neutralised or removed, the target cells which have been infected can be detected by the appearance in the culture of host cell nucleic acid or other intracellular markers. This detection method may be used, for instance, in place of reporter
15 bacteria in the bacterial detection assay described in WO92/02633. It can also be used in conjunction with the virus breeding method described herein, to allow early detection of infected target cells and thereby further discrimination between efficient and
20 inefficient virus. The target cells containing the most rapidly multiplying virus will be detectable first.

In one particular embodiment, the detection method employs ethidium bromide (EtBr) or analogues
25 eg. fluorochrome in the culture medium. EtBr binds to nucleic acid and when bound, its fluorescence in ultra violet light increases (Kosarev and Puchkov, 1989). Plaques of virally infected cells in a culture plate are easily visible in the presence of EtBr; an
30 automated system is also envisaged. Interestingly, extracellular viral particles which have been neutralised do not obscure results; it is thought that Composition A, an anti-viral composition described herein in Example 1, destroys viral nucleic acid so
35 that none binds the EtBr.

There are many applications in which the

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invention will be useful. Phage with improved attachment and infectious properties, and increased specificity towards a specific host cell, can be employed in the bacterial detection method described in WO92/02633. In that method, a chosen phage will infect and multiply only when brought into contact with its specific host cell. The amplified phage are then allowed to infect reporter bacteria and thereby produce an observable signal. Phage produced by the method according to the present invention, having a broader range of host affinity than wild type phage, may also be useful in bacterial detection assays.

Other applications of the invention include phage for use in bio-therapy for bacterial infection in humans or animals. Phage progeny can be selected for high killing levels. Advantages of using phage for destroying bacterial pathogens include continued destruction of the bacteria once excreted, such as Salmonella in poultry, and prevention or reduction of the spread of antibiotic resistance. The invention also opens up new possibilities for vaccine development. There are additional opportunities in the use of improved bacteriophage to control bacterial and other contamination by bioremediation eg. bacterial slime in paper production.

The invention will now be further described in the following Examples.

References

- Pelczar, M.J., Chan, E.C.S. Krieg, N.R., Edwards D.D. and Pelczar, M.F. (1993) Viruses: morphology, classification, replication. In Microbiology concepts and applications Part VII, Chapter 15, 401-435 pp. McGraw-Hill, INC. New York.

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Kosarev, N.V. and Puchkov, E.O. (1989) Determination of bacterial concentration using ethidium bromide fluorescence. *Mikrobiologiya*, 58, 126-130.

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EXAMPLES

Example 1

Improvements in phage attachment/infection for Felix 01 (wild type) to S.stanley, S.agona, S.infantis, S.kubacha, S.togba; and some Pseudomonas NCIMB bacteriophages to NCIMB and wild Ps.aeruginosa strains are resolved by adopting a two-step approach. All of these microorganisms are available to the public.

15

Preparation of Composition A

a. Preparation of 4.3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in Lambda-buffer (6mM Tris-HCl pH7.2, 10mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 $\mu\text{g/ml}$ gelatin).

20

First freshly prepare stock solution (0.53%) of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.053 gm ferrous sulphate in 10 ml Lambda-buffer). After it has been sterilised by membrane filtration (0.45 μm , Whatman) prepare the final ferrous sulphate concentration of 4.3 mM by transferring 4.1 ml of the ferrous stock solution into sterile test tube containing 14 ml of Lambda-buffer.

25

b. Preparation of Pomegranate Rind Extract (PRE).

Blend pomegranate rind in distilled water (25% w/v) and boil for 10 min. Centrifuge (20,000 x g, 4°C, 30 min), and autoclave supernatant (121°C, 15 min) and cool. Further purify the extract to a molecular weight cut-off of 10,000 Da by membrane ultra filtration, and store at -20°C. Other plant extracts which can be prepared in the same way as PRE and used successfully in the method described below are extracts

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of Viburnum plicatum leaves or flowers, maple leaves and commercial tea leaves. Ultrafiltration of these extracts is not necessarily required: storage is as for PRE.

5 c. Preparation of 13% PRE:

Mix 1.3 ml of stock solution of PRE (25% w/v) with 8.7 ml of Lambda-buffer.

Composition A was prepared 1-2 min prior to use by mixing 16.74 ml of 4.3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (a; yellow) with 8.3 ml of 13% PRE (b; yellow). After about 30 sec the colour of the mixture (a and b) changed greenish then to black. These mixtures of ferrous sulphate and PRE (a and b) should be protected from light.

15

Stage 1.

Stage one was based on the bacteriophage protection technique, as published in W092/02633; in brief: mix 10 μl of phage suspension (approximately 1×10^{12} pfu/ml) with 10 μl of bacterial cells suspension (10^9 cfu/ml) and incubate at 37°C for 15 min. Add 144 μl of Composition A (prepared as described above) to bacteria-bacteriophage mixture and, after 2 to 3 min incubation at room temperature, add the neutraliser (166 μl of 2% (v/v) Tween 80 in Lambda-buffer), followed by 100 μl of the same target bacteria and 2.3 ml of Tryptose Phosphate Broth (TPB; Oxoid) supplemented with 0.4% (w/v) agar and 0.0001% (w/v) ethidium bromide (EtBr). After 3 to 5 h incubation at 37°C observe and count the plaques under UV light.

30

In general, those phage which are capable of infecting their target bacterial cell within 15 min have escaped the kinetic-killing process of Composition A and create a visible "fluorescent" plaque in the top layer agar. Extracellular phage which have failed to infect the target bacteria are

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killed. The number of plaques obtained from the first round of this protocol are between 5 to 20 pfu of phage from an initial phage titre of 10^{12} pfu/ml. The phage plaques were collected as one plaque per 100 μ l of Lambda-buffer (6mM Tris, 10mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 μ g/ml gelatin; pH 7.2) and incubated for 4 h at 4°C.

Stage 2.

The second stage involves preparing a large volume of the corresponding phage by the sloppy layer plaque technique as follows: A 100 μ l sample of the stage 1 phage (10-fold serially diluted with Lambda-buffer) is mixed with 100 μ l of an overnight TPB culture of the appropriate target bacteria in a sterile Eppendorf micro-centrifuge tube and incubated for 15 min at 37°C. To each Eppendorf tube is then added 2.3 ml of 'sloppy agar' (TPB supplemented with 0.4% (w/v) agar), which has been melted and cooled to 40°C in a water bath. The contents of each tube are then well mixed by swirling, poured over the surface of a plate of tryptone phosphate agar (TPA) and allowed to set for 15 min at room temperature. The plates are incubated for 18 h at 37°C, and a plate showing almost confluent plaques used to prepare a concentrated phage suspension by overlaying with 5ml of Lambda-buffer (titre 10^{12} pfu/ml).

Example 2

Detection of S.stanley and S.agona using the wild type phage Felix 01 and bred Felix 01.

Initial difficulties of phage Felix 01 attachment to S.stanley were resolved by adopting the breeding phage method described in Example 1. Table 1 shows that wild type Felix 01 failed to detect S.stanley by failure to produce visible plaques. After

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breeding, however, phage Felix 01 gives a close parity between pfu and cfu when the bacterial cells are grown at 30-37°C for 18 h (Table 2). Plaque formation for Felix 01 is clearly dependent on the culture incubation temperature.

Similarly, breeding of Felix 01 on S.agona yields phage which gives a pfu to cfu relationship under the conditions used above (Table 3). Wild type Felix 01 give no plaques on S.agona.

Table 1. Detection of Salmonella stanley using wild type phage Felix 01.

Approximate cfu	0	10	100	1000	10,000
pfu	0	0	0	0	0

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Table 2. Effect of incubation temperature for 18 h culture of Salmonella stanley on plaque formation with a bred Felix 01 phage.

5	Approximate		Approximate	
	c f u		p f u	
10	<u>20°</u>		<u>25°C</u>	
	<u>30°C</u>		<u>37°C</u>	
	0	0	0	0
	10	0	6	9
	100	0	36	87
15	1000	0	260	900
	10,000	113	105	almost lysed
	100,000	220	792	lysis
	1000,000	lysis	lysis	lysis
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Table 3. Detection of S. agona using bred Felix 01 phage. Incubation at 37°C.

25	Approximate cfu		
	0	8	80
30	pfu		
	0	7	76

Note: "Non-bred" Felix 01 gave no plaques.

CLAIMS:

1. A method of obtaining from a virus population comprising a test virus improved virus progeny of the test virus, which method comprises the steps of:
 - (i) providing a target cell sample which comprises target cells of the test virus;
 - (ii) incubating a mixture of a sample of the virus population with the target cell sample so as to effect attachment to and infection of at least one target cell by at least one test virus particle, the conditions being controlled in a manner to ensure that a desired proportion of the test virus does not infect the target cells;
 - (iii) incubating the target cells to complete infection by the test virus and to cause virus progeny to be released; and recovering the virus progeny.
2. A method as claimed in claim 1, wherein the improved virus progeny has increased efficiency of infection of the target cell compared with the test virus.
3. A method as claimed in claim 1 or claim 2, wherein the test virus is a bacteriophage and the target cell is a bacterium.
4. A method as claimed in any one of claims 1 to 3, wherein an additional step between steps (ii) and (iii) comprises neutralising the extracellular virus with an antiviral agent.
5. A method as claimed in any one of claims 1 to 4, wherein incubation time in step (ii) is controlled to ensure partial but incomplete infection by the virus of the target cells.
6. Virus progeny of a test virus, wherein the virus progeny have improved properties compared to the test virus, following one or more rounds of

selective virus breeding.

7. Virus progeny obtained by the method according to any one of claims 1 to 5.

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INTERNATIONAL SEARCH REPORT

Internat. Application No

PCT/GB 95/00409

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N7/00 //C12Q1/70, A61K35/76

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 02633 (AMERSHAM INTERNATIONAL PLC) 20 February 1992 cited in the application see page 6, line 8 - line 19; claims 3,7,8 ---	1-7
Y	GB,A,2 060 690 (CORNING GLASS WORKS) 7 May 1981 see page 1, line 27 - line 50; claims 1-7,12 ---	1-7
Y	US,A,2 851 006 (W.I. TAYLOR ET AL.) 9 September 1958 see column 2, line 60 - column 3, line 18 --- -/--	1-7

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Ryckebosch, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB,A,771 653 (MEDICO BIOLOGICAL LABORATORIES LIMITED) see page 1, left column, line 43 - right column, line 2; claim 1 see page 1, right column, line 89 - line 93 -----	1-7

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Information on patent family members

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GB-A-771653		NONE	